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which was identified as HT-2,4-deacetylneosolaniol, 3'hydroxy HT-2, 3'hydroxy T-2 triol, and several more-polar unknowns. These T-2 metabolites are excreted from liver via bile into the intestine. Within 5 days, 75% of the total radioactivity was excreted in urine and feces at a ratio of 4 to 1. The appearance of radioactivity in the excreta was biphasic ( $T_{1/2,A}=2.2$  hr, 1.5 days and 8.2 hr, 1.7 days, for urine and feces, respectively). Metabolic derivatives of T-2 excreted in urine were T-2 tetraol, 4-deacetylneosolaniol, 3'hydroxy HT-2, and several unknowns. These studies showed a rapid appearance in and subsequent loss of radioactivity from tissues and body fluids. However, radioactivity ( $10^5$  dpm) was still detectable in tissues at 28 days. The distribution patterns and excretion rates suggest that liver and kidney are the principal organs of detoxication and excretion of T-2 toxin and its metabolites.

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### Fate and Distribution of <sup>3</sup>H-Labeled T-2 Mycotoxin in Guinea Pigs <sup>1,2</sup>

C. C. Perry, E. E. Kettner, M. T. Hurren, E. E. Dinterman, C. Matsen.

E. C. Hauer, and R. W. Wannenmacher, Jr.

United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

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T-2 toxin is a potent cytotoxic metabolite produced by the *Fusarium* species. The fate and distribution of [ $^3\text{H}$ ]-labeled T-2 toxin were examined in male guinea pigs. Radioactivity was detected in all body tissues within 30 min after an im injection of an  $\text{LD}_{50}$  dose (1.04 mg/kg) of T-2 toxin. The plasma concentration curve of radioactivity versus time was multiphasic, with an initial absorption half-life ( $T_{1/2,A}$ ) of less than 6 min. The initial half-life of elimination ( $T_{1/2,E}$ ) was 1.8 hr. Bile contained a large amount of radioactivity which was identified as HT-2, 4-deacetylneosolaniol, 3'-hydroxy HT-2, 3'-hydroxy T-2 triol, and several more-polar unknowns. These T-2 metabolites are excreted from liver via bile into the intestine. Within 5 days, 75% of the total radioactivity was excreted in urine and feces at a ratio of 4 to 1. The appearance of radioactivity in the excreta was biphasic ( $T_{1/2,A} = 2.2$  hr, 1.5 days and 9.2 hr, 1.7 days, for urine and feces, respectively). Metabolic derivatives of T-2 excreted in urine were T-2 tetraol, 4-deacetylneosolaniol, 3'-hydroxy HT-2, and several unknowns. These studies showed a rapid appearance in and subsequent loss of radioactivity from tissues and body fluids. However, radioactivity ( $10^3$  dpm) was still detectable in tissues at 28 days. The distribution patterns and excretion rates suggest that liver and kidney are the principal organs of detoxication and excretion of T-2 toxin and its metabolites.

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T-2 toxin, (4 $\beta$ ,15-diacetoxv 8 $\alpha$ -(3-methylbutyryloxv)-3 $\alpha$ -hydroxv- 12,13-epoxytrichothec-2-ene), is a toxic metabolite produced by the *Fusarium* species (Samburg et al., 1968a; Ueno, 1977, 1980). T-2 is a potent inhibitor of eukaryotic protein synthesis (Ueno et al., 1975; McLaughlin et al., 1977), as are most of the 12,13-epoxy-3 $\alpha$ -trichothecene compounds. These compounds are cytotoxic to cultured animal cells (Ueno et al., 1975; Thompson and Wannemacher, 1982), skin tissue (Samburg et al., 1968b; Ueno et al., 1979), and actively dividing cells (Saito and Ohtsuka, 1974), with varying toxicities related to their chemical structures (Ueno, 1977).

Several laboratories have reported on the metabolism of T-2 toxin in animals. Mice and rats metabolize T-2 to HT-2, neosolanolol, and several unknowns (Matsumoto et al., 1978), while broiler chickens metabolize the toxin to T-2 tetraol, 4-deacetylneosolanolol and several unknowns (Chi et al., 1978; Yoshizawa et al., 1980). In the lactating cow (Yoshizawa et al., 1981, 1982), T-2 is metabolized to HT-2, neosolanolol, 4-deacetylneosolanolol, 3-hydroxv T-2, and 3-hydroxv HT-2. These studies show that T-2 may be metabolized into products of equal or lesser toxicity (Ueno, 1977).

Because T-2 toxin is rapidly metabolized, previous studies were concerned with the first 48-72 hr period after the administration of the toxin. In cases of human toxicoses, samples are often obtained at much later time points (Mirocha et al., 1981). For this reason we conducted a comprehensive study of the metabolism of T-2 over 26 days. Specifically, we determined the absorption, distribution, metabolism, and excretion patterns of radioactivity after a single i.v. injection of [ $^{14}$ C]-labeled T-2 toxin in the guinea pig. In so doing we hoped to determine which of the major metabolites, detected in body fluids, could be used as clinical indicators

of trichothecene intoxication.

#### METHODS

**Reagents.** T-2 toxin was purchased from Calbiochem-Behring<sup>®</sup>. Purity was determined to be 99% by thin layer chromatography (TLC) and by gas chromatography-mass spectrometry (GC/MS). Standards for TLC (T-2, HT-2, T-2 triol, and T-2 tetraol) were purchased from Calbiochem. T-2 toxin was labeled with tritium in the C-3 position by New England Nuclear<sup>®</sup> using the method of Wallace et al., (1977). The purity (93% radiochemical purity, specific activity 8.9 Ci/mmol of «T-2) was determined by HPLC as well as TLC.

**Toxicity of T-2 mycotoxin in guinea pigs.** To establish the toxicity of an im injection of T-2 mycotoxin in the guinea pig, six (6) guinea pigs were injected with 3.0, 2.5, 1.75, 1.5, 1.25, 1.0, 0.75, or 0.5 mg/kg of T-2 toxin in ethanol:glycerol:water (2:3:5). The LD<sub>50</sub> was 1.04 +/- 0.25 mg/kg (mean +/- SEM) with a mean time to death of 16.6 hr (Pace et al., 1983).

**Treatment of Animals and Sample Collection.** One hundred and twenty five male guinea pigs <sup>7</sup>, weighing between 450 and 500 g, were injected im with 0.5 ml/kg of a solution containing 2 mg of T-2 toxin and 200 µCi of non-exchangable [<sup>3</sup>H]-T-2 toxin per ml in ethanol:glycerol:water (2:3:5) (LD<sub>50</sub>) (Pace et al., 1983). In the first study, twelve toxin treated guinea pigs were housed in stainless steel metabolic cages, and food and water intake were measured over the 28 day post-exposure time period. Urine and feces were collected daily from those guinea pigs that survived the toxin challenge. In the second study, guinea pigs were housed in groups of 12 each and allowed food and water *ad libitum*. Six guinea pigs were killed at 0.5, 3, 6, 12 hr; 1, 2, 3, 7, 14, and 28 days. Guinea pigs were

selected at random for necropsy for histopathological analyses. The guinea pigs were anesthetized with 0.5 ml/kg of a 1:1 mixture of Ketamine and Rompun® (1a) and blood was removed by cardiac puncture. Bile was collected from the gall bladder and urine collected from the bladder. Tissue samples, including liver, kidney, heart, lung, spleen, adrenals, testes, brain, stomach, large and small intestines muscle, and fat pad were collected, weighed and quickly frozen between liquid nitrogen-cooled clamps.

**Determination of metabolites.** Plasma, urine and bile were analyzed for radioactivity in a liquid scintillation counter\*. An aliquot (10-20  $\mu$ l) of plasma, urine, and bile was analyzed by TLC to separate and identify the toxin metabolites. Precoated silica gel TLC plates<sup>12</sup> (20 x 20 cm, 0.25 mm thick) were developed using two sequential solvent systems: (1) chloroform:ethylacetate:ethanol (50:25:25), (2) chloroform:ethylacetate:ethanol (80:10:10) (Pace et al., 1983). Mycotoxin standards were visualized based on a chromogenic reaction between 4-(p-nitrobenzyl) pyridine and the 12,13-epoxy group (Takitani et al., 1979) and samples were scanned for radioactivity with a Bioscan BID100 radioisotopic scanner<sup>13</sup>. The radioactive zones then were scraped, extracted with boiling ethyl acetate, and filtered through a glass wool plug. The filtrates were evaporate under a stream of nitrogen. The residues were derivatized with 1 drop each of methylene chloride and trifluoroacetic anhydride, sealed, and allowed to stand at room temperature 1.5-2 hr before nitrogen removal of the liquids. The residues were dissolved in a minimum of acetone and analyzed immediately by GC/MS using a Hewlett Packard 5995 B<sup>12</sup> equipped with a 25m X 0.2 mm ID fused silica capillary column (cross-linked OV-1, 11  $\mu$ m thick) interfaced directly to the source. Source temperature was 200°C, and GC conditions were



160°C, 1 min, 20°C/min to 250°C. Electron-impact spectra were run using 70 eV.

*Distribution.* To determine the tissue distribution of radioactivity, a weighed sample was oxidized<sup>13</sup> and the radioactivity determined in a liquid scintillation counter. Metabolites have not yet been determined in tissues.

*Kinetic Analysis.* The absorption and elimination of T-2 toxin were described by means of kinetic parameters,  $K_A$  and  $K_E$ , in the following equation:

$$C_T = A(-e^{-K_A T} + e^{-K_E T})$$

where  $C_T$  = concentration in plasma at time  $T$ ,  $A$  = constant,  $K_A$  = absorption constant,  $K_E$  = elimination constant. The semilog plot of the concentration against time is a straight line with  $K_E$  as the slope (Karlog et al., 1978). The concentration curve is divided into absorption and elimination phases with half-lives related to the absorption and elimination constants as follows:  $T_{1/2,A} = \ln 2/K_A$  and  $T_{1/2,E} = \ln 2/K_E$ .

*Statistical analysis.* The coefficient of variation,  $C = s/\text{mean}$ , was used to describe the amount of variation in the data. The standard deviation ( $s$ ) expressed as a percentage of the mean was less than or equal to 15%.

## RESULTS

### *General Observations.*

In guinea pigs receiving an LD<sub>50</sub> dose of T-2 toxin, food intake was markedly reduced but slowly returned to normal by day 7. This reduction in food intake combined with the intoxication resulted in an average loss of body weight of 73 g within 3 days. By 3 hr post-exposure, the guinea pigs

started to develop hypothermia; their temperatures reached a nadir of 32°C by 12 hr. Hematological and morphological changes were similar to those described previously by de Nicola et al. (1978).

#### *Time Dependence of Radioactivity in Blood, Urine and Feces.*

The plasma concentration curve (Fig 1, inset) was multiphasic with an initial absorption half-life of less than 5 min and an initial elimination half-life of 1.6 hr (Fig 1). The final elimination ( $k_{el}$ ) had an apparent half-life of 50 hr (Fig 1, inset). The appearance of radioactivity in the excreta was biphasic with initial absorption half-lives of 2.2 hr in urine and 8.2 hr in feces. The appearance of label in urine correlated with its disappearance from blood (2.2 hr vs. 1.6 hr).

The cumulative excretion of radioactivity in the urine and feces of the guinea pig is shown in Figure 2. In a five day period, 75% of the total radioactivity was excreted in the urine and feces at a ratio of 4:1 (Fig 2, left vertical axis). Radioactivity peaked in urine 24 hr after injection of the toxin and rapidly decreased over the next 4 days; while in feces, the radioactivity slowly increased over the first 5 days. At 28 days radioactivity ( $5 \times 10^4$  dpm) was still detectable in the excreta.

#### *Analysis of Metabolites in Bile and Urine.*

The bile, obtained from the gall bladder, had the highest specific activity (Table 1) and after 12 hr it contained 17% of the total administered radioactivity (Figure 3). The biliary metabolites were identified by radioisotopic scanning of a TLC plate<sup>14</sup>, illustrated in Figure 4, and structural assignments were made on the basis of electron impact mass spectra of their GC-separated trifluoroacetate derivatives. Standards (T-2,

HT-2, and T-2 tetraol) were run under the same conditions as were the unknown samples. Other structural assignments were based on the mass spectral library provided by Mirocha and Pawlosky (personal communication). 4-Deacetylneosolaniol (Yoshizawa et al., 1980) was the major metabolite (42 µg/ml) along with HT-2 (4 µg/ml), 3'-hydroxy HT-2 (Yoshizawa et al., 1982) (7.5 µg/ml, in a ratio of 5:3), and 3'-hydroxy T-2 triol (Cole et al., 1991) (9 µg/ml). Dehydration of the 3'-hydroxyl has been shown to occur readily under the trifluoroacetylation conditions (Pawlosky et al., 1984). Thus, the presence of the 3'-hydroxy metabolites was deduced from mass spectra of mixtures of the respective 2' and 3'-unsaturated compounds, separated by GC. A typical mass spectrum, that of the 2'-isomer resulting from 3'-hydroxy T-2 triol is depicted in Figure 5. The base peak at  $m/z$  83 is due to the conjugated ester moiety at C-8, and the fragments above  $m/z$  200 are characteristic of 3,4-dihydroxy-8,15-oxygenated 12,13-epoxytrichothec-9-ene trifluoroacetates (Pawlosky et al., 1984). In addition, a substantial quantity (21 µg/ml) of unidentified trichothecene-related polar metabolite(s) was found, and is under structural investigation. By 24 hr the concentrations of all biliary metabolites had decreased 4-fold. Figure 6 is a representative radiochromatogram of the urinary metabolites of T-2. At 3, 6, 12, 24, 72 hr the major urinary metabolites, identified by GC/MS, were T-2 tetraol (3.6 to 6 µg/ml), 4-deacetylneosolaniol (1.4 to 7 µg/ml), 3'-hydroxy HT-2 (3 to 4 µg/ml) and several more-polar trichothecene metabolites (4.7 to 7 µg/ml). The radioactive peak located at the origin was composed of several metabolites and comprised 5-20% of the total measured radioactivity. After treatment of the material that remained at the origin with  $\beta$ -glucuronidase, two additional less-polar radiolabeled peaks were separated by HPLC. This suggests that at least one of the components of this peak was conjugated as

glucuronide. By 26 days T-2 tetraol was the only identified T-2 metabolite. At all time points the urinary level of unmetabolized T-2 was below detection limits ( $\mu\text{g/ml}$ ).

#### Distribution of Radioactivity.

Recovery of total administered radioactivity, excluding carcass (skin and bone) associated counts, ranged from 74-107%. Radioactivity peaked in the bile at 12 hr, and in the large intestine at 24 hrs (Fig. 3). Radioactivity appeared in the large intestine in two phases: a rapid increase in the first 3 hrs, followed by a slow increase to 60% of the total administered radioactivity over the next 24 hr. After day 7, less than 1% of the total radioactivity remained associated with the GI tract. Figures 7 and 8 show the distribution of label into major tissues. The peak radioactivity in most tissues appeared at 30 min, then rapidly declined. While the total counts associated with the kidney (Fig. 7) were less than those with the liver, the specific activity (dpm/mg of tissue) was higher in kidney (Table 1). The specific activities of the heart, brain and testes were among the lowest measured.

Muscle and fat deposits (Fig. 8) contained the highest total counts. The values for the percentage of radiolabel in muscle and fat are approximates based on muscle being 40% of the total body weight and fat being 13% of the total body weight. The specific activity in muscle was comparable to that found in heart (Table 1).

#### DISCUSSION

Radiolabeled T-2 toxin was distributed in all tissues within 30 min

after an im injection of the toxin. The rapid incorporation of label suggests that toxic effects could begin shortly after exposure to the toxin. Except in the large intestine and bile, the radiolabel peaked by 30 min and rapidly declined, with no measurable long-term accumulation. In general, the early time (12-24 hr) distribution patterns paralleled the distributions reported using chicken (Chi et al., 1978; Yoshizawa et al., 1980) and swine (Robison et al., 1979) models, with a slightly higher concentration attributable to bile and the GI tract.

Our study represents a comprehensive investigation of the distribution and metabolism of T-2 toxin past the 72 hr time point. The plasma concentration curve shows that radioactivity can be detected in plasma as early as 6 min and as late as 28 days after exposure to the toxin. Even at the earliest time point (30 min), we were unable to detect unmetabolized T-2 (detection limit, 1µg/ml), suggesting that the long-term toxic element is a metabolite of T-2, possibly HT-2. The elimination phase of the plasma concentration curve appeared to be multiphasic due to the presence of toxin metabolites.

The distribution and excretion patterns suggest that the liver is the major organ for metabolism and detoxication of the toxin. By 30 min, the liver rapidly metabolized T-2 to HT-2 which, along with several more-polar metabolites, was eliminated from the liver via the bile. This is supported by the fact that the specific activity of the bile was highest at every time point. The bile, therefore, plays an important role in toxin elimination. Since bile was collected at death and not continuously, we could not determine how much radioactivity actually passed through the liver and bile. However, liver perfusion studies (Pace and Watts, 1983) suggest that during a single pass through the liver, 70% of the total administered radioactivity is extracted, and 50% appears in the bile.

The time dependent progression of peak radioactivity from bile (12 hrs) to large intestine (24 hrs) to feces (4 days) suggests that metabolites undergo enterohepatic circulation. The slow elimination of radioactivity from the intestine might account for the reported histopathological lesions in the GI tract of rodents (Brennecke and Neufeld, 1982). These studies suggest that enteric absorbants, such as charcoal, may be of some benefit in the treatment of T-2 intoxication.

Urinary metabolites peaked at 30 min and, over the 28 day study, 60% of the total radioactivity could be accounted for in urine. In contrast, in a study using orally administered toxin (Matsumoto et al., 1978) most of the radioactivity was excreted in the feces. The major urinary metabolite was T-2 tetraol, the toxicity of which is less than T-2 toxin (Cole et al., 1981). The data suggest that the quantitation of T-2 tetraol in urine may be a good index of exposure to the toxin.

TLC and GC/MS analysis of the urine and bile revealed the presence of several unknown T-2 metabolites which accounted for approximately 40-50% of the total radioactivity. The metabolic profile suggests that T-2 toxin is rapidly metabolized to HT-2 which is then converted to one of three metabolites as follows:

HT-2	T-2 tetraol via 4-deacetylneosolaniol (Yoshizawa et al., 1980)
HT-2	3'hydroxy HT-2 (Yoshizawa et al., 1982)
HT-2	3'hydroxy T-2 triol via T-2 triol or 3'hydroxy HT-2

The data presented here suggest that further characterization of the T-2 tetraol and polar metabolites in urine will yield valuable information regarding the metabolism of T-2 toxin.

In this study we investigated the absorption, distribution, metabolism,

and elimination of I-2 toxin and its metabolites in guinea pigs. The guinea pig efficiently converted I-2 to HF-2, which was further detoxified to polar metabolites. The detection of polar metabolites in urine as long as 25 days after toxin administration suggests that long term effects of I-2 toxin may be caused by its metabolic products.

#### FOOTNOTES

1. Portions of this work have been presented in abstract form: Fed Proc 42: 616, 1967; Fed. Proc. 42:1849, 1967.

2. The views of the authors do not purport to reflect the positions of the Department of the Army, or the Department of Defense.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Its facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

3. To whom correspondence should be addressed.

4. US Army Medical Biotechnology Research and Development Laboratory,  
Frederick, Maryland 21701

5. Calbiochem-Behring, San Diego, Calif. 92112

6. New England Nuclear, Boston, Mass. 02118

7. Hartley Strain: Buckberg Lab Animals

8. Haver Lockhart, Shawnee, Kansas 66201

9. Mark III Scintillation Counter, Searle Analytic, Inc., Des Plaines, Ill  
60018

10. Pre-coated silica gel thin layer plates, EM Science, Cincinnati,  
Ohio 45212



11. Bioscan, Inc., Washington, D.C. 20007

12. Hewlett Packard, 11000 Wolfe Rd., Cupertino, Calif. 95014

13. Model 306 Analyzer, Packard, Downers Grove, Ill 60515

14. TLC standards and identified metabolites had  $R_f$  values of 0.86

(T-2), 0.87 (HT-2), 0.54 (T-2 triol), 0.56 (olefin of 3-hydroxy HT-2), 0.47

4-deacetylneosalantol and olefin of 3-hydroxy T-2 triol, and 0.42 (T-2

metabolite).

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## LEGENDS

Figure 1. Semilog plot of the concentration of radioactivity in the plasma against time (first 24 hrs) after an im injection of labeled T-2 toxin.

Inset illustrates a multi-phasic plasma concentration curve over the 28 day study.

Figure 2. Excretion of radioactivity after administration of labeled T-2 toxin to guinea pigs, in terms of total cumulative excretion (●) and daily excretion in urine (○) and feces (■).

Figure 3. Distribution of radioactivity in the GI tract (stomach (■), large intestine (○), and small intestine (□) and bile (●) of guinea pigs after a single im administration of labeled T-2 toxin. Each point is the average of data obtained from 6 guinea pigs. The coefficient of variation was less than 15%.

Figure 4. Representative radiochromatogram of bile collected from the gall bladder of guinea pigs administered labeled T-2 toxin. TLC standards (T-2, HT-2, T-2 triol and T-2 tetraol) are indicated above the scan.

Figure 5. Mass spectrum of the olefin from 3-hydroxy T-2 triol.

Figure 6. Representative radiochromatogram of urine from guinea pigs administered labeled T-2 toxin. TLC standards (T-2, HT-2, T-2 triol, and T-2 tetraol) are indicated above the scan.

Figure 7. Distribution of radioactivity in liver (●), kidney (○) and heart (■) of guinea pigs after a single im administration of labeled T-2 toxin. Each point is the average of data obtained from 6 guinea pigs.

Figure 8. Distribution of radioactivity in muscle (●) and fat (○) of guinea pigs after a single im administration of labeled T-2 toxin. Each point is the average of data obtained from 6 guinea pigs.

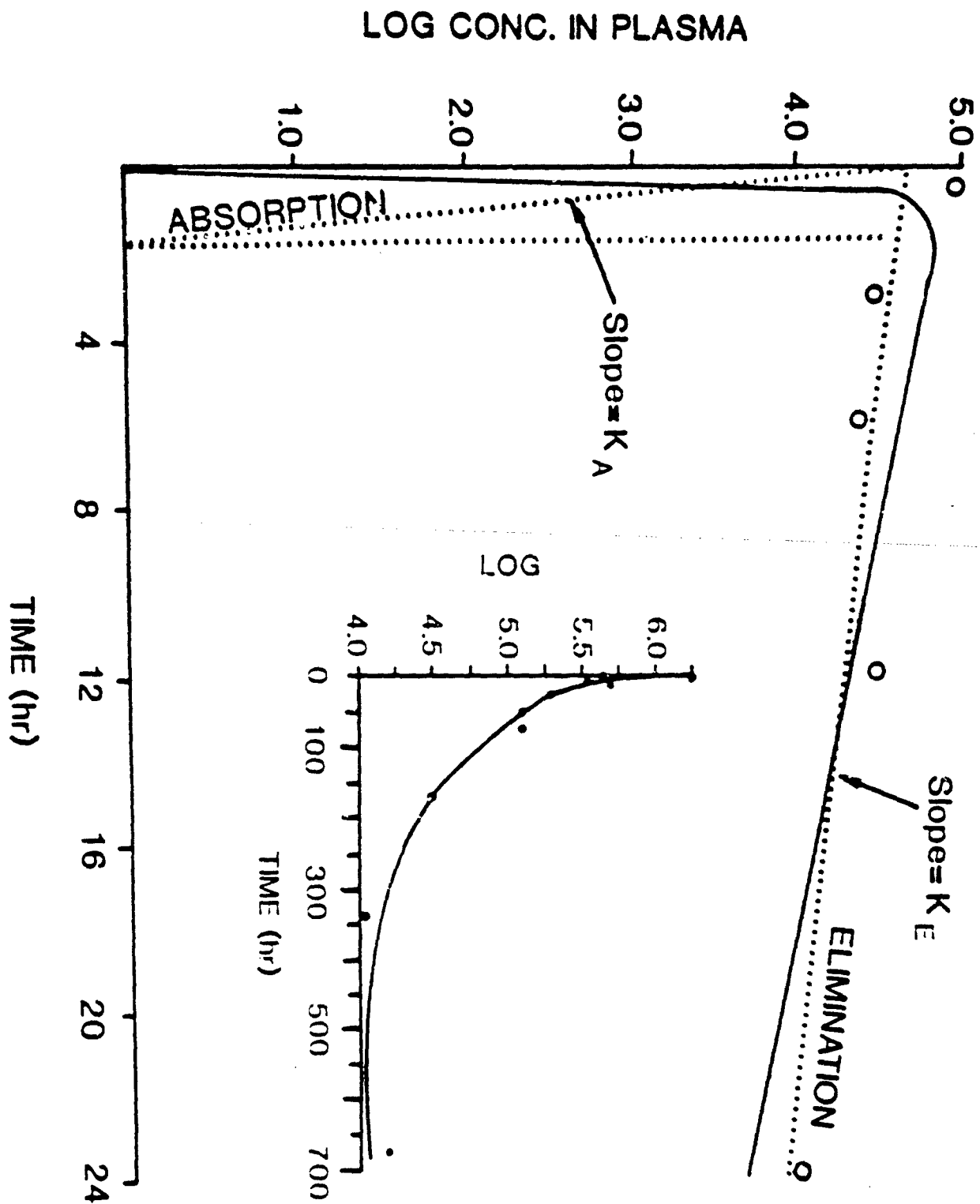
Table 1. Specific radioactivity in tissues of guinea pigs after a single in administration of labeled T-2 toxin\*

Tissues	Specific radioactivity (dpm/mg wet weight)								
	Time (hr)								
	0.5	3	6	12	24	48	168	336	672
Kidney	275.3	87.5	123.0	92.1	12.5	7.6	1.2	0.7	0.6
Liver	197.9	99.6	54.6	54.8	16.0	10.3	1.2	0.9	0.4
Lung	141.3	50.5	32.8	28.5	9.0	5.6	1.2	0.5	0.3
Spleen	134.0	58.6	37.3	29.7	9.5	5.6	1.6	0.9	0.6
Adrenals	131.3	28.6	16.6	13.5	6.6	4.6	2.9	2.9	2.4
Fat	120.5	54.5	42.5	16.9	8.6	3.0	1.5	1.0	0.3
Heart	114.8	24.0	14.0	7.3	5.1	3.0	0.4	0.3	0.2
Muscle	74.9	44.1	31.6	23.4	7.0	5.6	1.3	0.3	0.1
Testes	57.7	45.2	36.3	40.1	15.1	4.1	0.4	0.4	4.3
Brain	36.5	16.9	7.3	9.8	4.1	2.6	0.9	0.2	0.2
Plasma <sup>b</sup>	94.9	29.6	23.7	29.7	9.6	7.1	2.0	0.7	0.6
Bile <sup>c</sup>	247	281	538	55142	16738	6998	30	3.8	1.0

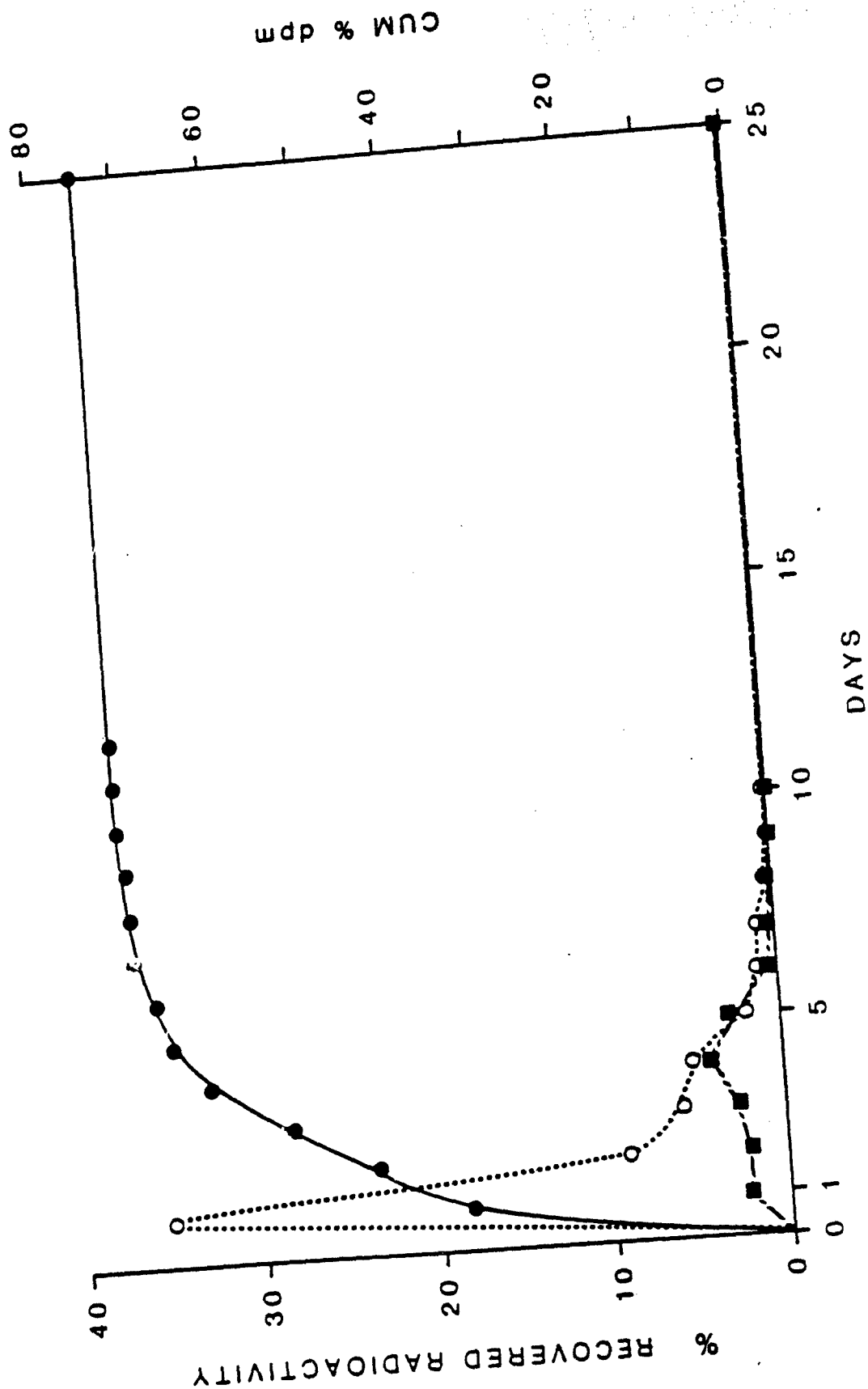
\*Mean values for six guinea pigs. The coefficient of variation was less than 15%. Tritium-labeled T-2 was administered at a dose of 1 mg/kg or  $2.36 \times 10^6$  dpm/kg body weight.

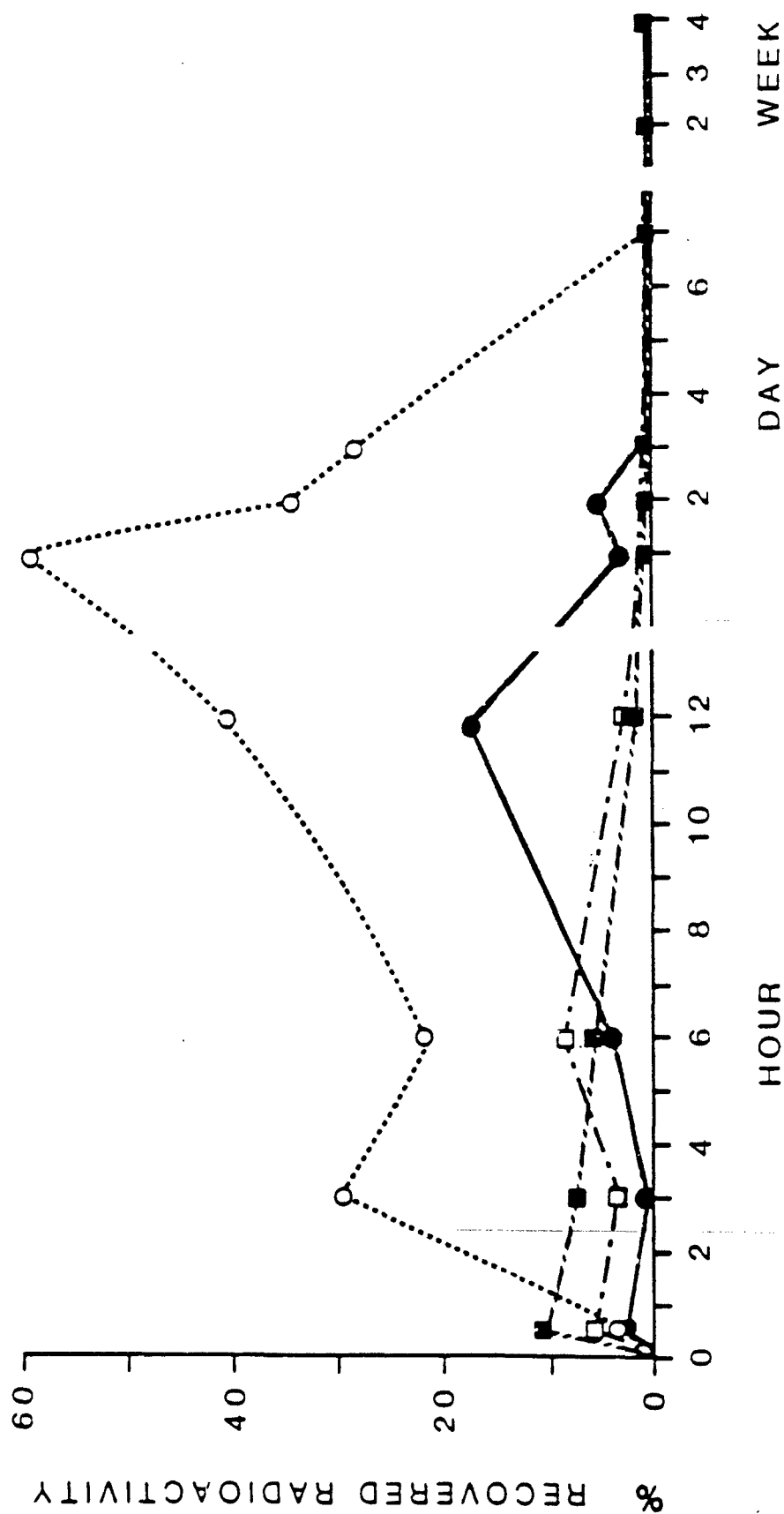
<sup>b</sup>Specific activity of plasma is expressed in units of dpm/ml.

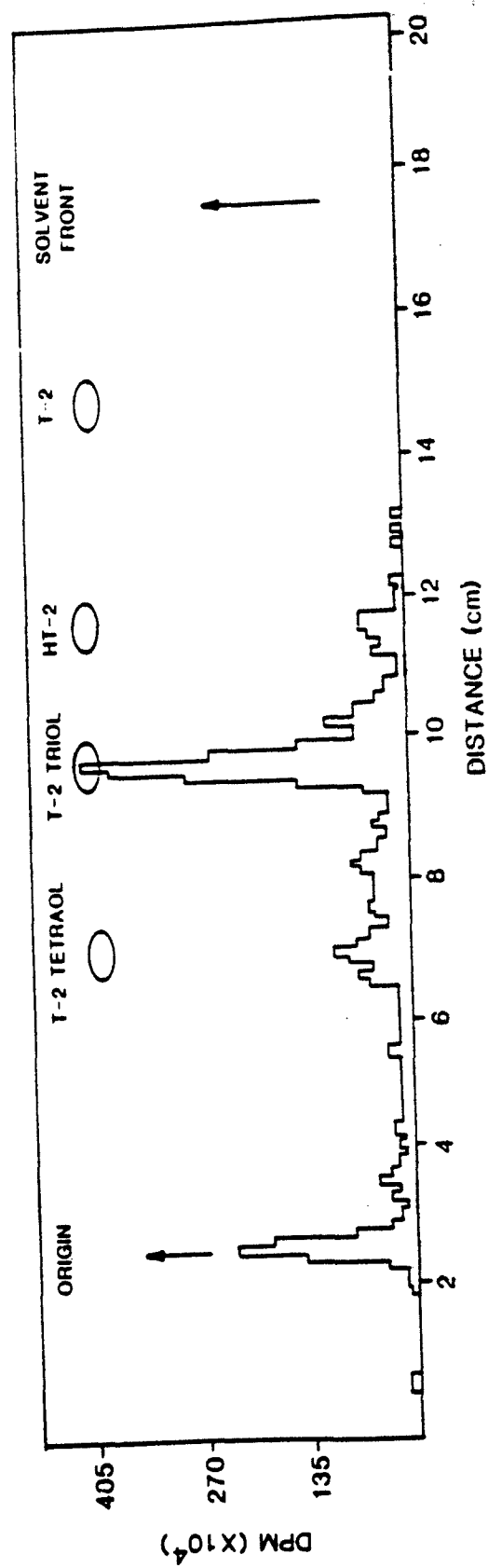
<sup>c</sup>Specific activity of bile is expressed in units of dpm/ $\mu$ l.

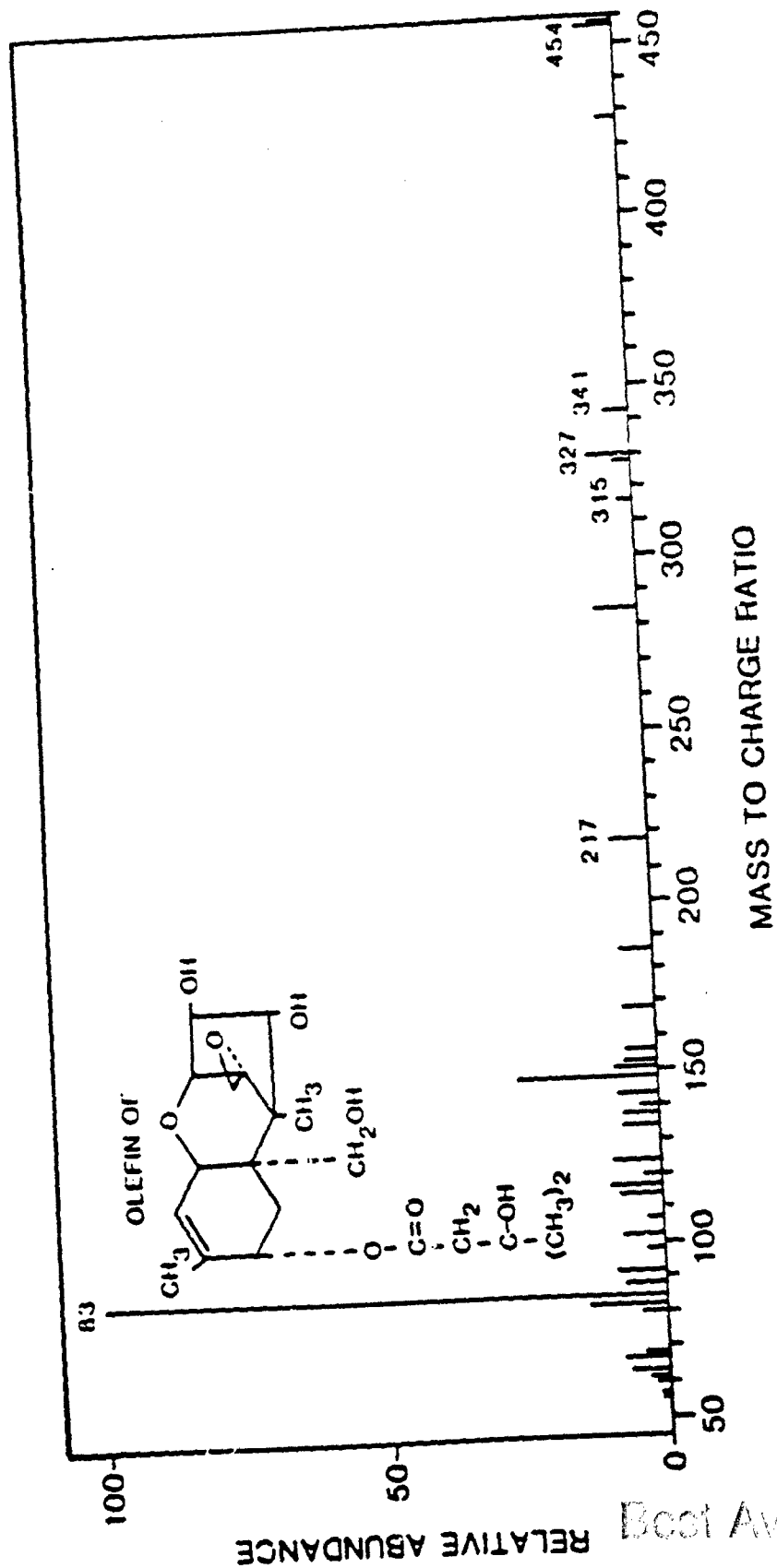


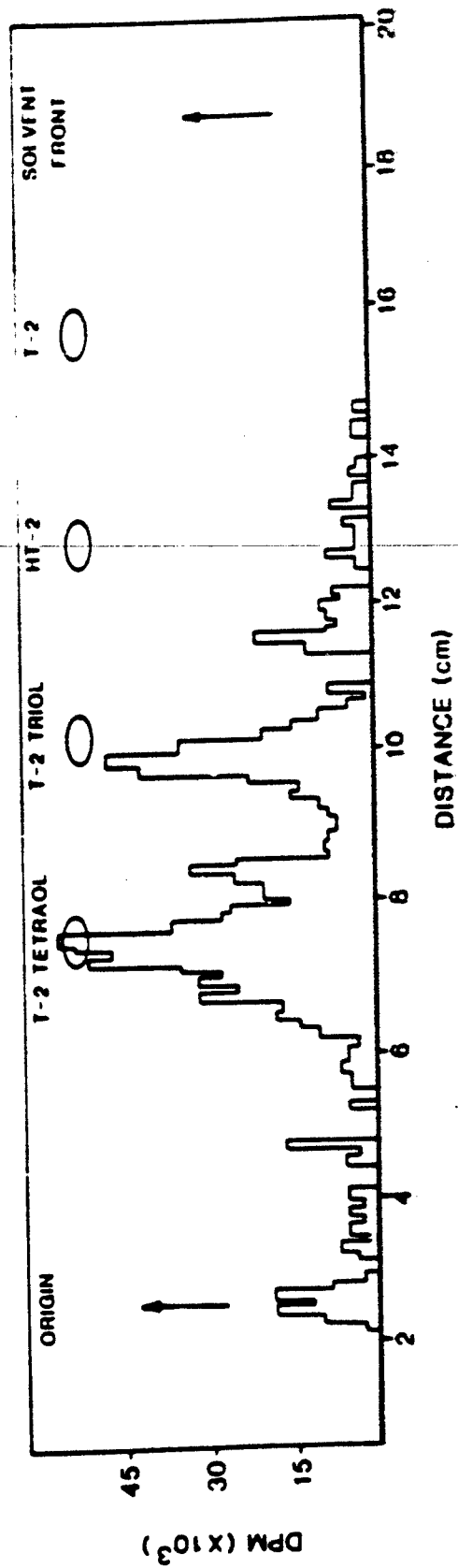


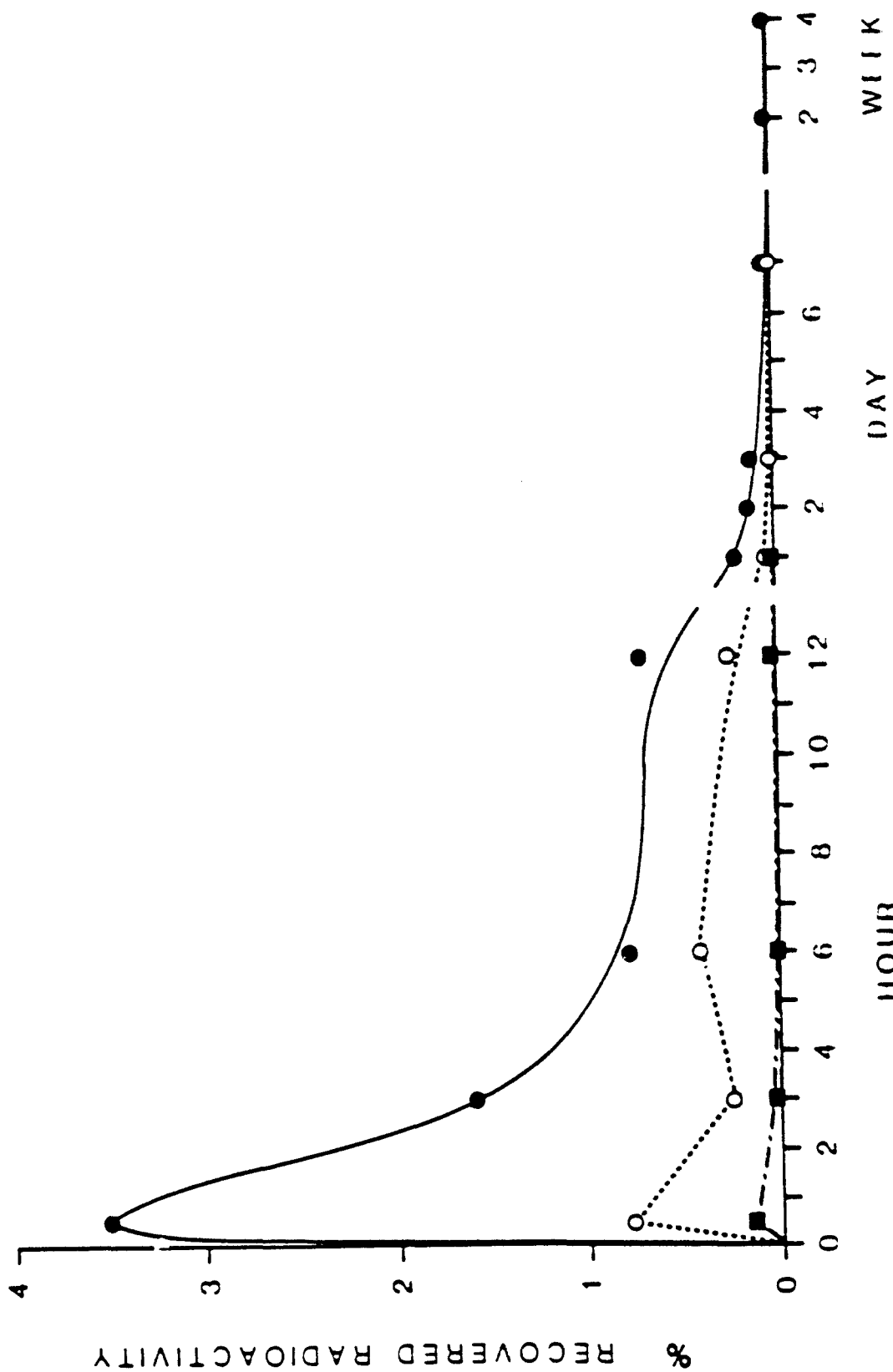




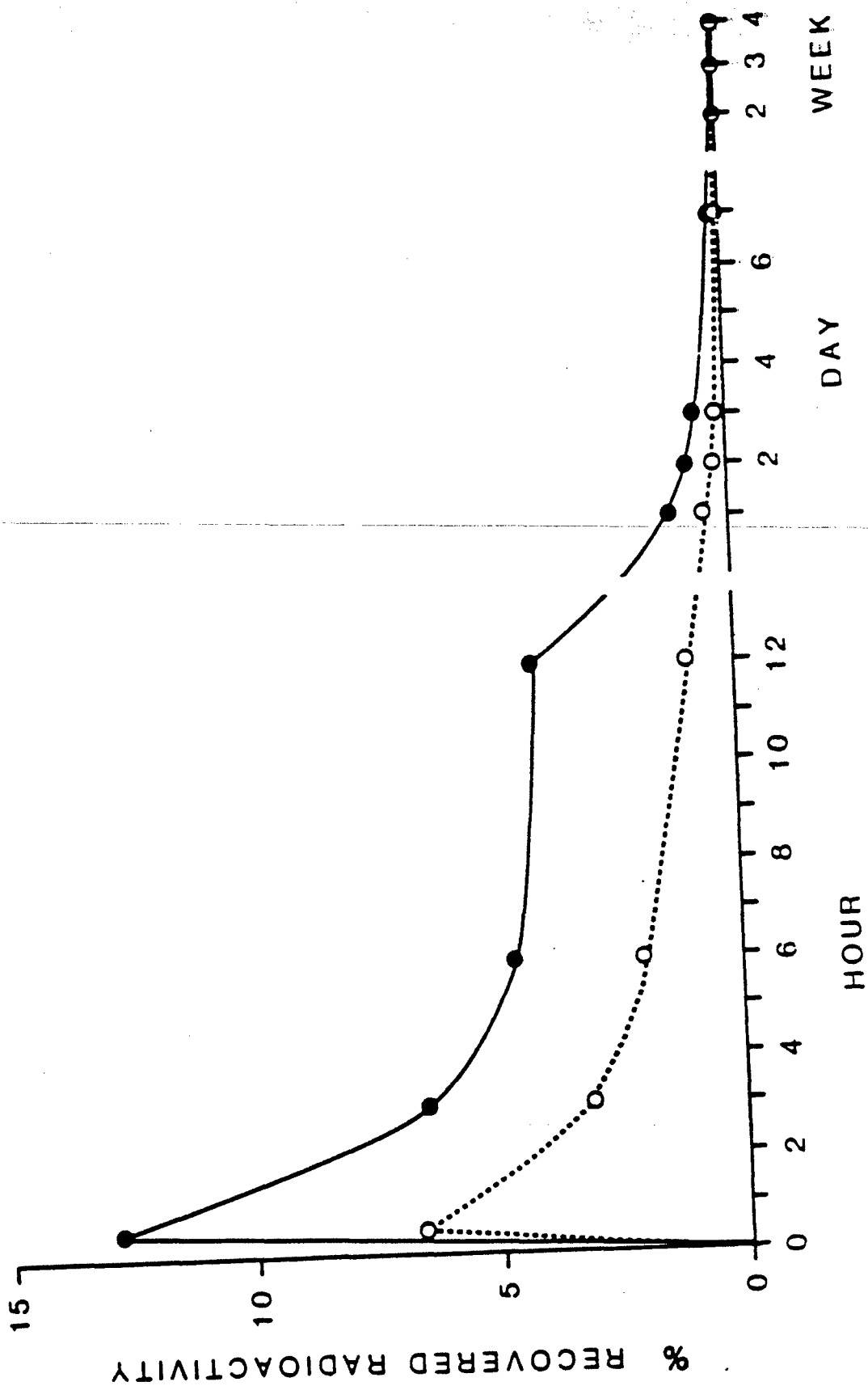








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